Neotropical Ichthyology, 14(1): e140158
DOI: 10.1590/1982-0224-20140158

Journal homepage: www.scielo.br/ni
Published online: 30 March 2016 (ISSN 1982-0224)

Inseminating dose and water volume applied to the artificial fertilization of *Steindachneridion parahybae* (Steindachner, 1877) (Siluriformes: Pimelodidae): Brazilian endangered fish

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The *Steindachneridion parahybae* is an endangered catfish from Brazil and strategies applied for gametes optimization are necessary. The aim of this study was to assess inseminating doses and water volume upon the fertilization, hatching rates and percentage of normal larvae in *S. parahybae*. Was used a randomized design in factorial scheme (4×4) with four inseminating doses: 1.0×10^4, 1.0×10^5, 1.0×10^6, 1.0×10^7spermatozoa oocyte^{-1} and four volumes of water: 1, 35, 65 and 95mL of water g^{-1} of oocytes. The combination of doses and volumes were performed in triplicates (n=48). Each incubator (1.5L of useful volume) with 1g of oocytes was considered as an experimental unit. Significant interaction between inseminating doses and volumes of water to the values of the fertilization rates and quadratic effect of doses and volume for the values of hatching rates were observed. The doses and volumes did not influence the percentage of normal larvae (87.70±5.06%). It is recommended the use of 5.5×10^6 spermatozoa oocyte^{-1} and 1mL of water g^{-1} of oocytes during *in vitro* fertilization procedure. These results allowed us to develop new biotechnological strategies applied to the conservation of *S. parahybae*.

O *Steindachneridion parahybae* é um bagre ameaçado de extinção no Brasil e estratégias aplicadas para a otimização de gametas são necessárias. O objetivo deste estudo foi avaliar doses inseminantes e volume de água sobre os valores das taxas de fertilização, eclosão e larvas normais em *S. parahybae.* Utilizando-se um delineamento experimental casualizado em esquema fatorial (4×4), com quatro doses inseminantes: $1,0\times10^4$; $1,0\times10^5$; $1,0\times10^6$; $1,0\times10^7$ espermatozóides ovócito^{-1} e quatro volumes de água: 1; 35; 65 e 95mL de água g^{-1} de ovócitos. As combinações de doses e volumes foram realizadas em triplicatas (n=48). Cada incubadora (1,5L de volume útil) contendo 1g de ovócitos foi considerada como uma unidade experimental. Interações significativas entre doses inseminantes e volumes de água para os valores das taxas de fertilização e efeito quadrático das doses e do volume para os valores das taxas de eclosão foram verificadas. As dosagens e os volumes aplicados não influenciaram no percentual de larvas normais (87,70±5,06). Recomenda-se a aplicação de $5,5\times10^6$ espermatozoides ovócito^{-1} e a utilização de 1mL de água.g^{-1} de ovócitos no procedimento de fertilização artificial *in vitro*. Estes resultados permitiram desenvolver novas estratégias biotecnológicas aplicadas na conservação do *S. parahybae*.

Keywords: Artificial reproduction, Freshwater fish, Oocytes, Sperm, Surubim-do-Paraíba.

Introduction

Steindachneridion parahybae (Steindachner, 1877) (Siluriformes: Pimelodidae), the surubim-do-Paraíba, is a endemic catfish to the Paraíba do Sul River basin (Garavello, 2005). The fish stock of this species in the wild has decreased greatly since the 1950s (Caneppele *et al*., 2009; Hilsdorf & Petrere Jr, 2002; Machado & Abreu, 1952) mainly due to uncontrolled fishing and anthropic activities, such as dam construction and excess of pollution (Araujo *et al*., 2009). *Steindachneridion parahybae* is currently on the red list of Brazilian animals threatened with extinction (Caneppele *et al*., 2008), and is considered as regionally extinct in the state of São Paulo (PAN, 2011).

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Caneppele *et al*. (2009) conducted the artificial reproduction of the *S. parahybae* by means of inducing agents, using the dry method for artificial fertilization (Ihering, 1937). The authors verified the ovulation in a period of 250 degree-hours with 85% of fertilized oocytes. These preliminary results have been used in recovery programs for the local fish fauna (Araújo, 2011; Honji *et al*., 2009; 2011; 2012; Sanches *et al*., 2014; 2015; Lopes *et al*., 2015). However, more studies about gamete characterization and reproductive management during the process of artificial fertilization, as well as about the reproductive biotechniques in captivity are necessary for the preservation of this species. They may contribute directly to the rational use of gametes, increasing the success of the artificial propagation, like the application of effective inseminating doses (Rurangwa *et al*., 2004).

Inseminating doses are unknown to the *S. parahybae* and were applied successfully in other species of native South American fish, providing suitable conditions to promote maximum fertilization: *Salminus brasiliensis* (Sanches *et al*., 2009; Weingartner, 2010), *Rhamdia quelen* (Bombardelli *et al*., 2006), *Brycon insignis* (Shimoda *et al*., 2007) and *Piaractus mesopotamicus* (Sanches *et al*., 2011a). The injection of suitable inseminating doses during the procedures of artificial fertilization brings important implications, such as higher values of fertilization rates, optimization of gametes and broodstocks, and also serves as basis for cryopreservation programs, allowing the thawing of the exact amount of spermatozoa that is necessary to fertilize the desired amount of oocytes (Bombardelli *et al*., 2006; Chereguini *et al*., 1999; Fogli da Silveira *et al*., 1988; Rurangwa *et al*., 1998; Sanches *et al*., 2011a; Sanches *et al*., 2009; Yasui *et al*., 2009).

Thus, the objective of this study was to evaluate the effect of inseminating doses and the water volume on the values of fertilization and hatching rates, as well as normal larvae in *S. parahybae*, kept in earthen ponds.

Material and Methods

Place and broodstock selection. The experiment was conducted at the Hydrobiology and Aquaculture Station of São Paulo Energy Company – EHA/CESP, in the town of Paraibuna – São Paulo - Brazil (23°24'54''S; 45°35'52''W), using *S. parahybae* broodstocks captured in the wild (F0), but maintained in captivity, and first generations (F1) of the fish farm station.

The fish, kept in two earthen ponds (200m^2), were fed twice a day, at 08:00a.m. and 4:00p.m., receiving pre-determined amounts of extruded commercial feed for carnivorous fish, with 40% crude protein in the proportion of 3% of biomass day^{-1}, before of reproductive period.

During the reproductive period (October-February/2011-2012), brood stocks suitable for spawning in the tank after gentle pressure applied to the abdomen were selected. Males showing semen release and females with slightly bulged abdomen and which released a small amount of oocytes that were uniform in size (Caneppele *et al*., 2009) were chosen. In addition, a small amount of oocytes was withdrawn from the females via biopsy, using a 2.0mm catheter and suction. That amount of oocytes was immersed in Serra solution (Lemanova & Sakun, 1975), so that it was possible to verify the percentage of oocytes with germinal vesicle migration (≥65% of peripheral nucleus). Eight females (1,350±661g), four F0 and four F1, and six males F0 (1,659±550g) were selected. After the selection, the brood fish were transferred to the laboratory and kept in aquaria (500L) equipped with an aeration feature during 24h (time necessary to the hormonal induction and artificial reproduction procedure). The water quality during this time was evaluated (22.9±0.3°C, pH 7.6±0.6 6.8±0.1 mg of dissolved oxygen L^{-1}).

For future verification, voucher-specimens from the same local were collected and stored in the fish collection of the Zoological Museum of São Paulo University - MZUSP, with catalog number 100672 and 108433.

Hormonal induction. The hormonal induction of all females was conducted by means of injections of two doses of crude carp pituitary extract (CCPE): the first dose consisted of 0.5mg of CCPE kg^{-1}, and the second one, of 5.0mg of CCPE kg^{-1} at an interval of 12 hours, diluted in saline (0.9% NaCl), as proposed by Caneppele *et al*. (2009). The doses of hormone were injected intramuscularly in the upper portion of the dorsal fin. Based in fact that the CCPE does not provide increase on sperm production and quality (Caneppele *et al*., 2015), the males were not submitted to the process of hormonal induction.

Collection and evaluation of semen and sperm characteristics. For the semen collection, the fish were restrained, dried (cloth and paper towel), and submitted to abdominal pressure from head to tail. The first drop of semen was discarded to avoid possible contamination with urine. The rest of the semen was collected in graded test tubes (±0.1 mL), and then kept at room temperature (≈25ºC). The contamination of the samples with blood and urine was avoided at the moment of semen release.

Analyses of sperm concentration, morphological changes of the spermatozoa and sperm viability were conducted in accordance with Sanches *et al*. (2009). Furthermore, the computerized analysis of sperm motility was conducted as proposed by Sanches *et al*. (2010; 2013).

Two samples of semen mixing "pool" were used to measure sperm concentration. In each one, 5 µL of the semen diluted in 5,000 µL of buffered formol-saline were used, resulting in a dilution of 1:1,000. A Neubauer hematimetric chamber was used for the sperm cell count (spermatozoa. mL^{-1}) (Sanches *et al*., 2011b).

Three slides with spermatozoa stained with rose bengal (Streit Jr. *et al*., 2004) were prepared using the same semen fixed in formol-saline to assess the possible

morphological changes of the spermatozoa. In order to do so, the methodology employed by Streit Jr. *et al*. (2006) and Bombardelli *et al*. (2010) for slide making was adapted. 500µL of spermatozoa+buffered formol-saline (dilution 1:1,000 of spermatozoa: fixative) were kept in Eppendorf tubes (1.5mL), to which 10µL of stain were added. After homogenization, 10µL of fixative+spermatozoa+stain were placed on one end of the glass slide. The slide was then inclined at an angle of 45º so that the droplet could run to the other end. The slides were dried in open air, and then analyzed under light microscope (obj. 40×) (Hafez & Hafez, 2004), with the evaluation of at least 200 spermatozoa (CBRA, 1998).

Sperm viability was assessed by the staining method eosin-nigrosin (Blom, 1950), using 30µL of semen, 90µL of eosin yellow (3%) and 90µL of nigrosin (5%). After the slides had been processed, 200 spermatozoa (CBRA, 1998) were analyzed under light microscope (obj. 40×) (Sanches *et al*., 2009), where the stained spermatozoa were considered dead and the colorless ones were considered alive (Kavamoto & Fogli da Silveira, 1986).

The evaluation of sperm motility by the computerized method was carried out as described by Wilson-Leedy & Ingermann (2007) and adapted for neotropical catfishes by Sanches *et al*. (2010; 2013), using free software. The process of activation was conducted in Eppendorf tubes (1.5mL), using 1µL of semen and 1,000µL of water from the incubation system. The images were obtained by a Nikon trinocular light microscope (model e50i), previously focused (obj. 10×), attached to a Prosilica GE680c camera (*http://www.alliedvisiontec.com*), and connected to a computer (intel core i7© CPU 2.3 GHz, 4Gb Ram), operational system Microsoft Windows 7©. The videos were captured by the software AVTUniversalPackage at 101fps (640x480 pixels) in *.avi format, edited with the software VIRTUALDUB-1.9.0 (*virtualdub.org*), and exported as image sequence in *.jpg format to a specific directory. The corresponding images were opened, edited in IMAGEJ (*National Institutes of Health, USA*, *http://rsbweb.nih.gov/ij/*) and compiled by means of the plugin CASA (*University of California and Howard Hughes Medical Institute, USA, http://rsbweb.nih.gov/ij/plugins/casa.html*).

The videos were processed based on the description of the necessary components for the use of the application CASA via free software (Wilson-Leedy & Ingermann, 2006). However, the configurations used were adapted for the species (Sanches *et al.* 2013). The sperm parameters obtained from four replications were: motility rate (MOT), curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), straightness (STR) and progression (PROG).

The amount of motile spermatozoa per milliliter of semen was estimated based on the previous assessment of the sperm quality. These results were used to estimate the dosage of semen according to the amount of motile spermatozoa. Therefore, semen from only two males F0 (1,950±495g) that had presented over 80% of motile cells was used and mixed to form the "pool" of semen used in fertilization assay. This procedure was employed to avoid the negative effects on fertilization rate. The semen collected from other four males was discarded due to its poor quality.

Collection and evaluation of oocytes. The oocytes were collected after a period of 320 degree-hours (14h after second hormonal induction and water at 22.9°C), registered from the last hormonal injection onward (Caneppele *et al*., 2009; Okawara *et al*., 2015). Sample was performed by stripping, which consists of gentle abdominal massage in cephalocaudal direction. The oocytes were collected in a porcelain mortar, and then weighed (±0.1g) for the estimate of the number of oocytes produced per gram of female. However, for the estimate of the total number of oocytes in each experimental unit, four sub-samples of 1 gram of oocytes from each female were counted.

After the oocyte stripping, a subjective (macroscopic) quality check, with regard to size uniformity, yellow color and absence of blood was used as prerequisite. Thirty grams of oocytes from each female were then homogenized, originating a sample consisting of a pool of oocytes that was then used in the experiment of fertilization. Based in subjective characterization of high quality (size and uniform color, without blood and ovulation on same time), oocytes from only two females F0 (2,300±424g) were used. A batch of 1g of oocytes was immersed in Gilson solution (Simpson, 1951) for the assessment of oocyte diameter (stereomicroscope, 20×). For the fertilization, pre-established amounts of oocytes, chosen according to the weight (1g) were employed.

Experimental design. The inseminating doses (spermatozoa oocyte^{-1}) and water volume (mL g^{-1} of oocytes) employed were determined by three pilot experiments.

The selected gametes were distributed in a factorial experimental design 4×4. The treatments consisted of four inseminating doses of 1.0×10^4, 1.0×10^5, 1.0×10^6 and 1.0×10^7 spermatozoa oocyte^{-1}, four water volumes of 1, 35, 65 and 95mL of water g^{-1} of oocytes and three replications (48 experimental units). A conic hatchery (1.5L of useful volume) containing 1.0g (287±7) of fertilized oocytes with their respective inseminating doses (dosed with automatic micropipettes) and water volume was considered as one experimental unit. In order to minimize the effect of the size of the container on the water volume, the fertilizations were performed in different plastic cups with the following volumes: 50, 100, 150 and 200mL for the respective water volumes 1, 35, 65 and 95mL g^{-1} of oocytes.

Artificial fertilization. The artificial fertilization of *S. parahybae* oocytes was conducted using the dry method (Ihering, 1937) with gametes homogenization in the absence of water. The water of the artificial incubation system was added later for gamete activation, with the pre-established qualities and amounts described above.

After the artificial fertilization, the eggs were transferred to experimental hatcheries (1.5L) with enough water flow to keep the eggs moving, in a 300L system of water recirculation. The temperature, pH, oxy-reduction potential, electrical conductivity, dissolved oxygen, oxygen saturation and total dissolved solids were monitored by the device Horiba (Model U-50).

Eleven hours after the fertilization, corresponding to the end of the epibolic movement or closure of the blastoporus (Honji *et al*., 2012), the percentage of fertilized eggs was estimated based on the count of all the eggs from each experimental unit. The translucid eggs that presented an apparently normal embryonic development were considered as fertilized, and the unfertilized eggs (=addled) were opaque or white. After the eggs had hatched (1,320 degree-hours), the values of the hatching rates were estimated. In order to do so, all the newly hatched larvae from each experimental unit were anesthetized with benzocaine (50mg.L^{-1}) for 30 seconds, for later classification into normal and abnormal under stereomicroscope (10×).

Statistical analysis. The possible influence of the inseminating doses (ID) and water volume (WV) on the response variables (RV) was assessed by means of the response surface model:

$$RV=\partial_0+\partial_1 ID+\partial_2 WV++\partial_3(ID*WV)+\partial_4(ID)^2+\partial_5(WV)^2+\varepsilon$$

where: ∂i = constants; ε = error with $\sim N(0, \partial^2)$

The non-significant parameters (P>0.05) were progressively removed according to higher-order terms by the *backward stepwise method*. The assumptions were followed as suggested by Myers (1990) and Quin & Keough (2002). The analyses were conducted by means of the *Statistica*© software (Statsoft, 2005). The maximum lines were plotted in 3D and 2D graphs from partial derivatives of the statistical model in relation to the significant variables.

Results

Females released 73.55±7.00g of oocytes, corresponding to a fecundity of 9,418±2,610oocytes kg^{-1} of brood fish. Soon after the stripping, the oocytes exhibited an average diameter of 1.70±0.07mm. The water quality parameters assessed are shown in Table 1, and the semen and sperm parameters in Table 2.

Values of fertilization rate presented interaction effect (P<0.05) between the inseminating doses and applied volume of water (Fig. 1). The highest values could be verified in the application of the inseminating doses of 5.4×10^6 spermatozoa $oocyte^{-1}$ in 1.0mL of water g^{-1} of oocytes, up to 6.4×10^6 spermatozoa $oocyte^{-1}$ in 95.0mL of water g^{-1} of oocytes (Fig. 1). At intermediate volumes (x), the highest values of fertilization rate may be found with the inseminating dose (y) obtained by the equation y=5,423,431.90+10,674.42*x (Fig. 1).

Table 1. Measured parameters of the quality of the water used in the fertilization of oocytes and incubation of the *Steindachneridion parahybae* eggs (mean±standard deviation).

Parameters	Values
Temperature (°C)	22.96±0.41
pH	7.07±0.17
Oxy-reduction potential(ORPmV)	308.07±13.08
Electrical conductivity (m*S* cm^{-1})	0.04±0.03
Dissolved oxygen (mg L^{-1})	6.19±0.47
Oxygen saturation (%)	74.06±5.46
Total dissolved solids (g L^{-1} TDS)	0.03±0.02

Table 2. Semen and sperm parameters in *Steindachneridion parahybae* (mean±standard deviation).

Parameters	Values
Collected volume (mL)	26.80±7.07
Relative volume (mL kg^{-1})	14.68±7.35
Motility rate (%)	87.10±6.69
Curvilinear velocity (μm s^{-1})	132.71±11.99
Average path velocity (μm s^{-1})	103.65±11.32
Straight line velocity (μm s^{-1})	91.06±9.05
Straightness (%)	87.94±1.79
Progression (μm)	3.870±372
Sperm concentration (espermatozoa mL^{-1})	1.70×10^{10}
Sperm viability (%)	88.83±4.86
Normal spermatozoa (%)	52.49±1.54

The behavior of the values of hatching rate was similar to the behavior of the fertilization rate. However, no interaction between the inseminating doses and the water volume was verified, only quadratic effect (P<0.05) (Fig. 2). The highest hatching rates were found with the inseminating dose of 5.5×10^6spermatozoa $oocytes^{-1}$, independently of the water volume (Fig. 2). As verified for the fertilization rate, the lowest water volume promoted the highest hatching rates (P<0.05) (Fig. 2).

The percentage of normal larvae (87.70±5.06%) did not suffer influence of the dose and volume (P>0.05).

Discussion

The sperm production found in this study was higher than the one verified for the same species by Caneppele *et al.* (2015) and the computerized sperm characteristics were higher than the ones verified by Sanches *et al*. (2013) for fresh semen, and by Araújo (2011) for cryopreserved semen. Therefore, the results obtained in this study were considered satisfactory for artificial reproduction carried out in laboratory routines. Egg production and diameter were similar to the ones found by Caneppele *et al*. (2009), with production between 9,000 and 10,000 oocytes kg^{-1} of female and diameter around 1.90mm.

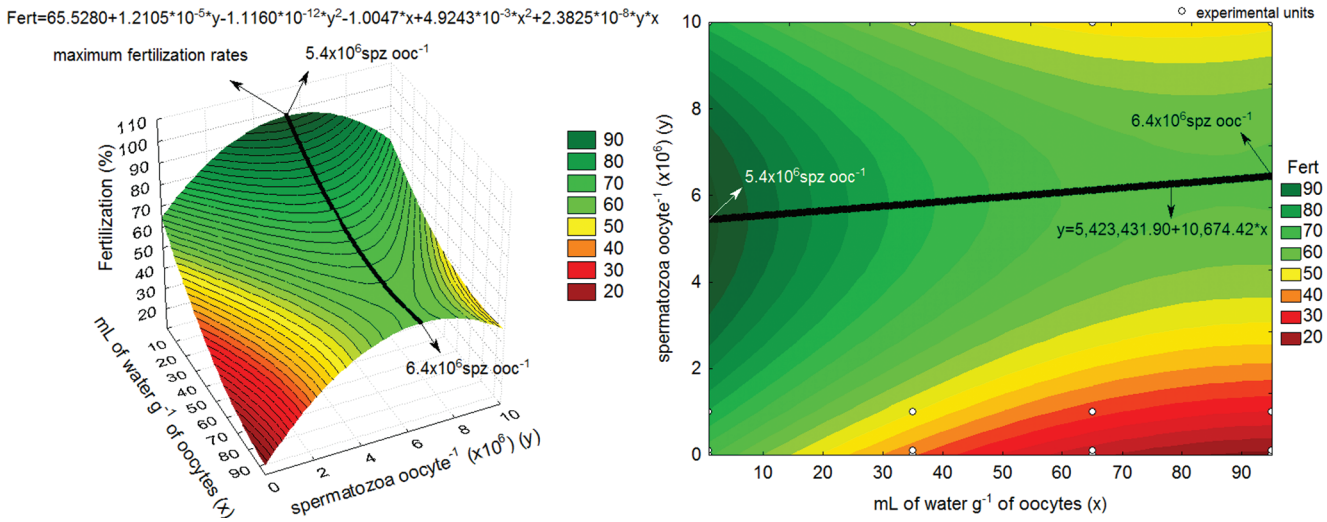

Fig. 1. Values of fertilization rates (Fert) of *Steindachneridion parahybae* with regard to different inseminating doses *versus* water volume. Left – 3D graphical representation. Right – 2D graphical representation. Spermatozoa oocyte^{-1} (spz ooc^{-1}). r^2=77.47%.

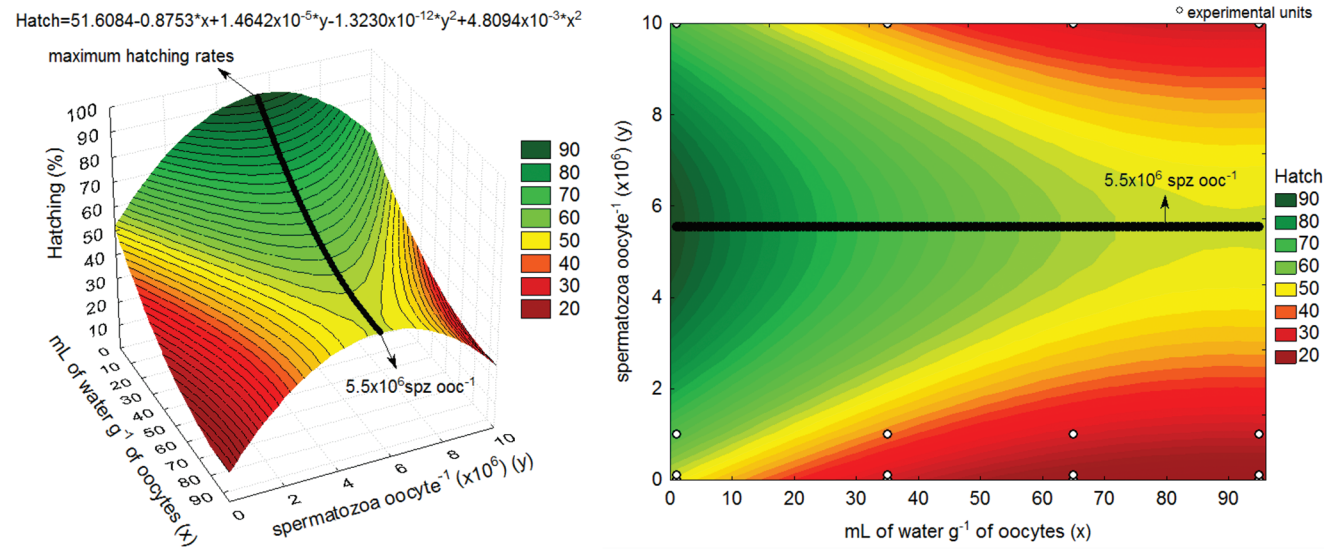

Fig. 2. Values of hatching rate (Hatch) of *Steindachneridion parahybae* with regard to different inseminating doses *versus* water volume. Left – 3D graphical representation. Right – 2D graphical representation. Spermatozoa oocytes^{-1} (spz ooc^{-1}).

The behavior of the fertilization and hatching rates observed in the present experiment was different from what had been observed in other species of native South American fish: for the *Brycon insignis*, Shimoda *et al*. (2007) verified a *plateau* behavior of the values of fertilization rate from the dosage of 3.1×10^5 spermatozoa oocyte^{-1} onward. Sanches *et al*. (2009), studying *Salminus brasiliensis,* observed quadratic behavior, with better doses in the application of 3.1×10^4 spermatozoa oocyte^{-1}. When studying the *Piaractus mesopotamicus*, Sanches *et al*. (2011a) observed influence in the values of fertilization rate only at super dosages of semen. The authors verified that the inseminating dose of 7.0×10^7spermatozoa oocyte^{-1} reduced the values of the rates when compared to the dosages between 7.0×10^3 and 7.0×10^6spermatozoa oocyte^{-1}. Authors also verified that the water volume influenced on the rates, since when there was little water in the procedure (0.5mL mL^{-1} of oocytes), it affected the values of fertilization rate negatively, and the use of 15 to 60mL mL $^{-1}$ of oocytes did not interfere with the results. The use of large amounts of water in procedures of artificial fertilization was also reported by Weingartner (2010) for *S. brasiliensis*, increasing the fertilization rate in four times in the application of 50 instead of 10mL 10g^{-1} of oocytes. The use of large amounts of water may help with the dilution of some toxic components, such as magnesium, calcium and potassium (from the ovarian fluid or the use of cryoprotectants), which at certain amounts may cause reduction of the sperm quality and consequently lower fertilization (Yasui *et al*., 2012).

We found that the use of large amounts of water negatively influenced the values of fertilization and hatching rates, and the volume of 1.0mL g^{-1} of oocytes was the most recommended. The use of large amounts of water might have diluted the inseminating medium excessively, so even with large amounts of spermatozoa, it might have provided an inappropriate condition for the sperm entry in the micropyle (Chereguini *et al*., 1999; Sanches *et al*., 2009).

Despite the fact that lower volumes have provided better fertilization rates, it is not recommended to use lower volumes than the minimum value tested in the present experiment (1.0mL g^{-1} of oocytes), because such volumes might compromise the sperm activation and later, the fertilization rates (Billard & Cosson, 1992).

Best values of fertilization and hatching rates were estimated with sperm dosages around $5.5x10^6$ spermatozoa $oocyte^{-1}$. However, ideal dosages may vary according to the quality of the gametes (Bobe & Labbe, 2010), and the procedure employed to achieve fertilization (Rurangwa *et al*., 2004).

The curvilinear velocity (VCL) and the average path velocity (VAP) are factors that may influence the fertilization of the *S. parahybae* (Araújo, 2011). This author observed a positive linear correlation between those velocities and the values of fertilization, indicating that faster spermatozoa present better chances of reaching the micropyle before its closure, or before their movement stops completely. Therefore, it is assumed that spermatozoa that exhibit circular movements show higher fertilization capacity. This hypothesis is corroborated by Tused *et al*. (2008), who verified positive correlation between the values of fertilization rate and VCL, and negative correlation with the straight line velocity (VSL) for *Oncorhynchus mykiss*. These results indicate that the lower the VSL, the higher the values of fertilization, since the VSL is indicative of straightness (positive correlation) (Tuset *et al*., 2008).

In general, the time of sperm activation in freshwater fish is short. This is therefore another factor that might influence the values of fertilization rate (Cosson, 2010). Caneppele *et al.* (2015), studying *S. parahybae,* observed that the time necessary for approximately 50% of the spermatozoa to lose their motility was 38.76±0.74 seconds, and that after around one minute all of them stopped moving. The use of specific activators may ensure longer time of sperm motility for the *S. parahybae* (Araújo, 2011). Nevertheless, their influence on the values of fertilization rate is unknown.

Furthermore, other factors may vary, such as oocyte diameter, because the larger the oocyte diameter, the more spermatozoa are necessary to surround the perimeter of the oocyte and reach the micropyle. That fact may be observed when compared with other species, such as *Rhamdia quelen,* which exhibits oocytes with average diameter from 1.0mm (Pereira *et al*., 2006) to 1.5mm (Amorim *et al*., 2009), 1,141oocytes g^{-1} and minimum inseminating dose of 8.9×10^4spermatozoa $oocyte^{-1}$ (Bombardelli *et al*., 2006). Sanches *et al*. (2010), studying the same species, observed the VAP of 58.67±9.01µm s^{-1} and progression of 1,202.80±163.89µm 15 seconds after activation, unlike *S. parahybae,* which despite exhibiting larger eggs, also exhibits higher values of VAP and PROG, and even so needs higher inseminating doses. Therefore, more studies are necessary to clarify those effects on the species.

The values of fertilization and hatching rates verified with the inseminating doses and water volumes tested in the present experiment revealed important effects on the artificial reproduction of the *S. parahybae*. New studies will be able to contribute to the reproductive efficiency for the utilization of genetic material, maximization of the broodstocks and use of cryopreservation programs. Furthermore, they will also contribute to studies on the characterization of gametes and their possible influence on fertilization.

In conclusion, the highest oocyte fertilization and egg hatching rates for *S. parahyba* may be obtained with the use of an inseminating dose of $5.5x10^6$ spermatozoa $oocyte^{-1}$, and water volume of 1.0mL g^{-1} of oocytes, corresponding to each 10g of oocytes, 10mL of water and 1.0mL of semen.

Acknowledgements

Authors would like to thank the employees of the Hydrobiology and Aquaculture Station of the São Paulo Energy Company (CESP) in the town of Paraibuna, São Paulo, Brazil and the São Paulo Research Foundation - FAPESP (grant number: 2009/18609-6 and 2010/02818-5), and the National Council for Scientific and Technological Development - CNPq (grant number 478347/2009-0).

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Submitted October 22, 2014
Accepted January 14, 2016 by Bernardo Baldisserotto